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Citation	Biochemical and Biophysical Research Communications, 417(1), 484-489 https://doi.org/10.1016/j.bbrc.2011.11.146
Issue Date	2012-01-06
Doc URL	https://hdl.handle.net/2115/48497
Type	journal article
File Information	BBRC417-1_484-489.pdf



Title: All-trans Retinoic Acid Inhibits the Recruitment of ARNT to DNA, Resulting in the
Decrease of *CYP1A1* mRNA Expression in HepG2 Cells

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Abstract

Aryl hydrocarbon receptor (AHR) and AHR nuclear translocator (ARNT) are well-conserved transcription factors among species. However, there are a very limited number of reports on the physiological function of AHR, particularly on the regulation of AHR by endogenous compounds. We hence investigated the effects of all-trans retinoic acid (atRA) on cytochrome P450 (*CYP*) *1A1* gene transcription as a model of AHR-regulated transcription mechanisms in HepG2 cells, a human hepatoma cell line. Treatment with atRA significantly reduced transactivation and expression of *CYP1A1* mRNA to less than half of its control value, and this inhibitory effect was mediated by RAR α . The result of chromatin immunoprecipitation assay indicated that treatment with atRA at 1–100 nM drastically inhibited the recruitment of ARNT to DNA regions containing xenobiotic responsive elements. In conclusion, atRA at physiological concentrations could reduce AHR-mediated gene transcription via the inhibition of recruitment of ARNT to relevant DNA regions.

Key words;

Retinoic acid, aryl hydrocarbon receptor, aryl hydrocarbon receptor nuclear translocator, recruitment

Abbreviations;

atRA: all-trans retinoic acid

AHR: aryl hydrocarbon receptor

ARNT: aryl hydrocarbon receptor nuclear translocator

ChIP: chromatin immunoprecipitation

CYP: cytochrome P450

DMEM: Dulbecco's modified Eagle's medium

PAS: Per-ARNT-Sim

RA: retinoic acid

RAR: retinoic acid receptor

RARE: retinoic acid responsive element

siRNA: small interference RNA

XRE: xenobiotic responsive element

Introduction

The aryl hydrocarbon receptor (AHR), a member of the Per-ARNT-Sim (HLH-PAS) family, is known to be a ligand-activated transcription factor [1]. In the absence of ligand, the AHR resides in the cytoplasm where it is associated with heat-shock protein 90 [2], the cochaperone protein p23 [3], and hepatitis B virus X-associated protein 2 [4–6]. After binding of ligand, the AHR translocates to the nucleus where it dimerizes with a protein known as AHR translocator (ARNT) [7, 8]. The dimer then recognizes its cognate DNA sequence, termed xenobiotic responsive element (XRE), and modulates expression of a battery of genes [1].

Most of our knowledge of AHR/ARNT-regulated transcription comes from studies of the induction of the *cytochrome P4501A1* (*CYP1A1*) gene by a wide spectrum of xenobiotic chemicals including polycyclic and halogenated aromatic hydrocarbons and aromatic amines, e.g. dioxins, 3-methylcolanthrene, and beta-naphthoflavone. AHR ligands have certain common characteristics with respect to their chemical structure; they are hydrophobic, planar, or coplanar molecules of polycyclic structure [9].

For a long time, researchers have made extensive efforts to identify an endogenous ligand of the AHR. Previous reports have proposed reliable evidence that tryptophan and its metabolites could act as endogenous agonists at the AHR and induce AHR-regulated gene transcription both *in vivo* and in cultured cells [10, 11]. In addition, there is evidence of a ligand-independent pathway that a variety of agents can transcriptionally activate *CYP1A1*, as measured by the AHR response, including hormones, omeprazole, and notably carotenoids, even though they lack the chemical characteristics typically associated with high AHR affinity [12–18]. However, there is no definitive explanation for the physiological function of AHR in the various tissues in which it is expressed, particularly when addressing the question of which endogenous compounds regulate AHR at physiological concentrations, the exception being tryptophan.

Our previous studies have reported that the AHR can be activated by carotenoids which are dietary constituents [17, 18]. Retinoids, the most abundant end products of carotenoids, are well known endogenous compounds which can exert various bioactivities via the retinoic acid receptor (RAR). However, there is little information on the effect of retinoic acid on AHR-mediated gene regulation. Therefore, we investigated the effects of all-trans retinoic acid (atRA) on gene regulation by the AHR. The physiological concentration of total retinoic acid (RA) is reported to be 30–100 pmol/g in rat and mouse liver [19, 20], and that of atRA is

reported to be around 11.3 pmol/g liver and 1.8 pmol/ml plasma in rats [21]. In the case of humans, the plasma level of atRA is in the range of 4–8 pmol/ml plasma [22]. The concentrations of atRA used in this study were similar to these physiological levels. Finally, we found evidence suggesting that atRA at physiological concentrations negatively regulates the constitutive expression of *CYP1A1* mRNA in human liver.

Materials and Methods

Chemicals

Dulbecco's modified Eagle's medium containing a high glucose concentration (DMEM, D5796) was purchased from Sigma Chemical Co. (St. Louis, MO, USA), trypsin-EDTA and all-trans-retinoic acid (atRA) from Wako Chemical Co. (Tokyo, Japan), fetal bovine serum (FBS) from Gibco Laboratories (Grand Island, NY, USA), dimethyl sulfoxide (DMSO) from Nacalai Tesque (Kyoto, Japan), and AGN193109 from Santa Cruz (Santa Cruz, CA, USA). Retinoic acid and AGN193109 were dissolved in DMSO and kept at -20°C in the dark.

Cell culture and treatment

The human hepatoma cell line HepG2 obtained from RIKEN Cell Bank (Tsukuba, Japan) was cultured in DMEM supplemented with 10% heat-inactivated FBS at 37°C in a humidified atmosphere of 5% CO_2 in air. When the HepG2 cells reached 80% confluence, the medium was replaced with fresh medium containing 1 nM–100 nM atRA or 0.1% DMSO as a vehicle control. We also investigated the effects of AGN193109 (a pan-RAR antagonist) on *CYP1A1* mRNA expression in atRA-treated cells. When HepG2 cells reached 80% confluence, the medium was replaced with fresh medium containing 0.2% DMSO or 2 μM AGN193109. After 1 h incubation at 37°C and 5% CO_2 , we added an equal volume of fresh medium

containing 0.2 DMSO as a vehicle control or 2–200 nM atRA. Final concentrations of each reagent were 1 μ M (AGN193109) and 1-100 nM (atRA), respectively.

Quantitative PCR

After incubation with atRA at 37°C and 5% CO₂ for 20 h, total RNA was isolated from cells using Tri Reagent (Sigma) according to the manufacturer's protocol. Total RNA concentration and quality were checked by using a Nanodrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Waltham, Massachusetts, USA). The RNA quality was estimated from the 260/280 and 260/230 nm absorbance ratios and confirmed by denaturing agarose gel electrophoresis. We synthesized cDNA from 500 ng of total RNA using a Takara Revetra kit (Takara, Ohtsu, Japan). The levels of *CYP1A1* mRNA were measured using an Applied Biosystems StepOne™ Real-Time PCR System (Foster, CA, USA), as described below. We employed the comparative CT quantification ($\Delta\Delta$ Ct) qrtPCR method to compare changes in *CYP1A1* gene expression. Relative quantification was performed using *Glucuronidase beta* (*GUSB*) as an endogenous control gene. The gene bank accession numbers, and Applied Biosystems assay IDs were, respectively: NM_000499.3 and Hs00153120_m1 (*CYP1A1*), and NM_000181.3 and Hs00939627_m1 (*GUSB*). An identical set of PCR cycle parameters was used for all genes: 95°C for 10 min, and 40 cycles at 95°C for 15 sec and 60°C for 30 sec, which was validated by Applied Biosystems.

Gene silencing with siRNA

Double-stranded siRNAs targeting human RAR α (GeneBank accession no. NM_000964) were purchased from Sigma Genosys. Negative control siRNA from Ambion (Austin, TX, USA) was used in control experiments. HepG2 cells were transfected with siRNAs at 50 nM using siPORT Neo FX Transfection Agent (Ambion), according to the supplier's protocol. Thirty-six hours after transfection, the media were replaced with fresh medium containing 0.1% DMSO or 100 nM atRA. After incubation at 37°C for 20 h, the expression levels of CYP1A1 and RAR α were measured by real time PCR as previously described.

Reporter plasmid construction

The pGL3-XRE promoter vector, a kind gift from Dr. Hines (Professor and Co-Director of the Birth Defects Research Center), harbors the 1.2kb 5' flanking region of human CYP1A1 in the pGL3 promoter vector (Promega, Madison, WI) [23]. We constructed pGL3-XRE reporter vectors possessing RARE1A1 mutations or XRE deletion mutations using the PrimeSTAR Mutagenesis Basal Kit (Takara) according to the manufacturer's protocol. The RARE sequence was mutated from AGGTCACCACGGGGCA to AGGTGACCACGGGACA which does not interact with RARs [24].

Luciferase assay

When HepG2 cells cultured in 60 mm dishes reached subconfluency, cells were transiently transfected with reporter vectors (native or mutated pGL3-XRE and pRL-SV40) using FuGENE HD (Roche Molecular Biochemicals, Indianapolis, IN, USA) and seeded into 96-well plates. After incubation at 37°C for 24 h, cell media were replaced with fresh medium containing 0.1% DMSO or 1–100 nM atRA. After 24 h treatment with atRA, cells were subjected to luciferase assays using the Dual-Glo Luciferase Assay System (Promega).

ChIP assay

HepG2 cells cultured in 100 mm diameter dishes were treated with 0.1% DMSO, 1 nM, or 100 nM atRA for 20 h. After incubation, formaldehyde (1% final concentration) was added to the media for 10 min to cross-link proteins with DNA. Cells were rinsed twice with ice-cold PBS, scraped into 1 ml ice-cold PBS containing a protease inhibitor cocktail, placed into tubes each containing 2×10^6 cells, and centrifuged at 3000 g for 5 min at 4°C. After removing the supernatants, cells were lysed with 300 μ l of lysis buffer (1% SDS, 5mM EDTA, 50mM Tris, pH 8.1, and protease inhibitor cocktail) on ice for 15 min. The solutions were then sonicated four times for 20 sec each time with a Sonifier 450A (Branson, Danburg, CT, USA, output: 1, duty cycle: 40%). Sonication has been shown to yield DNA fragments of 200 bp to 1 kb in length. The soluble chromatin was centrifuged at 16,000 g for 10 min at 4°C and 150

µl of aliquots of supernatant were transferred to fresh tubes. Each aliquot was diluted to 1 ml with dilution buffer (1% Triton X-100, 2 mM EDTA, 20 mM Tris, 150mM NaCl, and protease inhibitor cocktail). The soluble chromatin was immunoprecipitated with 1 µg of anti-AHR (Abcam, Ab84833), anti-ARNT antibody (Santa Cruz, sc-55526), or normal rabbit IgG (Santa Cruz, sc), using Dynabeads Protein G magnetic beads (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. DNA was eluted from the magnetic beads for 30 min at 65°C in 100 µl of elution buffer (1% SDS, 0.1M sodium bicarbonate, 0.2M NaCl), and protein-DNA cross-linking was reversed by incubation for 5 h at 65°C. Samples were digested with Proteinase K (0.2 mg/ml) for 1 h at 45°C. Isolated DNA was purified using a PCR purification kit (QIAGEN, Valencia, CA, USA) and eluted in 50 µl of DDW.

PCR for ChIP

ChIP DNA (5 µl) was amplified by PCR with primers 5' CGGTCCTTCTCACGCAAC 3' and 5' CTCCCGGGGTGGCTAGTGCTTTGA 3' (amplifying the region from 754 to 988 bp upstream of the human CYP1A1 transcription start site) by using the following cycles: 96°C for 5 min; 45 cycles at 96°C for 15 sec, 60°C for 30 sec, and 72°C for 30 sec in 10 µl of PCR reaction mixture: 0.25 U of Ex Taq (Takara), Ex Taq buffer, 100 µM dNTP mix, and 0.5 µM primer.

Statistical analysis

The mRNA expression results and relative transactivation abilities are expressed as the mean \pm SD. Statistical significance was assessed using a Student's *t*-test; differences between observations at the $p < 0.05$ level were considered statistically significant.

Results

Transactivation and *CYP1A1* mRNA expression were inhibited by atRA.

As shown in Figure 1, atRA decreased *CYP1A1* mRNA expression and transactivation of the native enhancer and promoter regions of human *CYP1A1* in HepG2 cells in a dose-dependent manner. In atRA-treated cells, there was a clear connection between the inhibition of *CYP1A1* mRNA expression and transactivation ability on the -1137 to +59 sequence of human *CYP1A1*. The transactivation ability on this region in cells treated with 10–100 nM atRA for 24 h decreased to approximately 60% of that in control cells; moreover, 10–100 nM atRA applied for 20 h decreased *CYP1A1* mRNA expression to less than 50% of its control.

***RAR α* was required for the inhibition of *CYP1A1* transactivation by atRA.**

As shown in Figure 2-A, the reduction of *CYP1A1* transcription by atRA was attenuated by AGN193109, a pan-antagonist at RARs. To confirm whether the inhibitory effect of atRA on *CYP1A1* transactivation was mediated by *RAR α* , a gene silencing experiment using small interference RNA (siRNA) was performed. Gene knock down with si*RAR α* reduced the expression of *RAR α* mRNA to approximately 45%, compared to that of cells transfected with siNC (Figure 2-B, left panel). As shown in Figure 2-B, right panel, atRA did not alter the expression levels of *CYP1A1* mRNA in the presence of si*RAR α* . These results indicated that

the reduction of *CYP1A1* transactivation by atRA was mediated by the RAR α .

The inhibition of *CYP1A1* transactivation by atRA was mediated via XRE, not RARE1A1.

A reporter assay was performed in HepG2 cells using two mutated-reporter vectors which possess human *CYP1A1* enhancer and promoter regions with either the four XREs sequences deleted (4 XREs-deleted) or with critical mutations to the RARE1A1 sequence (RARE-mutated). Mutations in the RARE1A1 sequence did not alter reporter activity in vehicle control cells, but significantly reduced it in the presence of atRA (Figure 3, left panel). Thus, it was suggested that RARE1A1 activation contributed to the positive regulation of the gene in the presence of atRA. In contrast, deletion of the 4 XREs decreased transactivation to the basal level, and this activity was not further modified by treatment with atRA (Figure 3, right panel). These results suggested that the negative regulation of *CYP1A1* transcription by atRA was dependent on the XRE sequence rather than RARE.

The recruitment of ARNT to DNA was inhibited by atRA.

To confirm the effects of atRA on the recruitment of transcription factors to the *CYP1A1* promoter region, we employed chromatin immunoprecipitation (ChIP) assay. HepG2 cells were treated with 0–100 nM atRA for 20 h and subjected to ChIP assay with antibodies

against AHR and ARNT. DNAs from immunoprecipitates were amplified by PCR using a primer set which recognized the -988 to -754 bp sequences from the human CYP1A1 transcription start site, which contains two XREs. We found that AHR and ARNT were recruited to the region in the absence of AHR ligand (Figure 4). Treatment with atRA had no effect on the recruitment of AHR to the region. However, the recruitment of ARNT was not detected in cells treated with 1 nM or 100 nM atRA for 20 h (Figure 4).

Discussion

The function of AHR in ligand-free states and the constitutive regulation of *CYP1A1* mRNA expression are poorly understood, whereas *CYP1A1* transactivation by ligand-bound AHR has been well investigated using typical AHR ligands, including dioxins and 3-methylcolanthrene. According to a previous report by Hestermann and Brown, after stimulation of AHR by beta-naphthoflavone, a potent agonist of the AHR, AHR rapidly binds with the promoter region of its target genes, such as *CYP1A1*, and recruits co-activators for histone acetylation, leading to the recruitment of RNA polymerase II [25]. The recruitment of transcription factors resulting from AHR-ligand binding is clearly organized in sequence and occurs almost instantaneously, leading to an explosive elevation of gene transcription.

Although the AHR requires either ligand-dependent or -independent activation for transformation to cell nuclei, ARNT is constantly located in the nucleus and is available to access to DNA. A previous report has indicated that ARNT can form homodimers, as well as heterodimers with AHR or hypoxia-inducible factors [26]. Combining these facts, we can highlight the importance of the role of ARNT in the constitutive expression of *CYP1A1* in the absence of a critical ligand of AHR.

The present study demonstrated that atRA at 1–100 nM inhibited the recruitment of ARNT to XRE-containing regions, which are important in the transactivation induced by ligand-activated AHR [25, 27], resulting in a decrease of the transcription of *CYP1A1*. We also found that atRA did not alter the protein expression of AHR or ARNT, and intracellular localization of AHR (data not shown). A previous study has also suggested an interaction between the transcription of *CYP1A1* and the RAR-mediated cascade based on the existence of a functional *cis*-element of RAR in the enhancer and promoter regions of human *CYP1A1* [28]. However, the inhibitory effect of atRA on *CYP1A1* mRNA expression observed in our study required RAR α , although the target site was not RARE but XRE, suggesting a new interaction mechanism among nuclear receptors. It has been reported that RA can suppress gene transcription by ligand-activated AHR through the formation of a physical interaction between the AHR and a co-repressor in Caco-2 cells [29]. It should thus be noted that gene transcription cascades can be indirectly modified through interactions among co-factors that are shared by nuclear receptors.

Recently, there has been growing evidence suggesting complicated interactions among cytosolic and nuclear receptors, including direct and/or indirect interactions mediated by co-activators and co-repressors. ARNT can act as a co-activator of estrogen receptors in the absence of AHR ligand, but ligand-activated AHR deprives ARNT from estrogen receptors,

leading to antiestrogenic effects [30]. However, the estrogen receptor suppresses gene regulation by AHR/ARNT through a direct protein-protein interaction with the complex [31]. On the other hand, Ross-Innes and colleagues have reported that RAR α is an essential component for maintaining of estrogen receptor-cofactors interaction [32]. In addition, it has been reported that RAR α and retinoid X receptor α can directly interact with MOP4 and CLOCK, which are classified to PAS proteins as well as AHR and ARNT, and can negatively regulate MOP4 and CLOCK-mediated transcriptional activation by RA in a concentration dependent manner [33]. These previous reports lead us to consider the possibility that ARNT, estrogen receptor, and RAR are co-localized in the nucleus, and that gene transcription mediated by any one of these factors may be modified by the others.

In summary, our current findings have indicated that atRA at physiological concentrations decreases the transactivation of *CYP1A1* not through an inhibition of the recruitment of AHR, but by an inhibition of recruitment of ARNT to XRE-containing regions. We now intend to undertake a detailed study of the mechanisms of RAR interactions with AHR and ARNT.

Acknowledgements

This project was funded in part by Japan Society for the Promotion of Science grants 220051870 and a Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, and Technology of Japan, awarded to M. Ishizuka (No. 19671001).

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Figure legends

Figure 1. Relative mRNA expression and transactivation of CYP1A1 were reduced to approximately 50 % by treatment with atRA.

Open diamonds: Cells were exposed to 0.1–100 nM atRA or 0.1% DMSO as a vehicle control. After 20 h incubation, the *CYP1A1* gene mRNA levels were measured by real time PCR using GUSB mRNA as an endogenous control. Filled diamonds: The pGL3-promoter vector containing the natural enhancer and promoter regions of human *CYP1A1* was transiently transfected into HepG2 cells with the control vector, pRL-SV40. After 24 h incubation in normal growth media, cells were treated with 0.1–100 nM atRA or 0.1% DMSO for 24 h. Each value represents the mean \pm SD of three wells. * indicates significance at the $p < 0.05$ level (Dunnett's *t*-test) between the Ct values of controls and atRA-treated cells.

Figure 2.

A; The reduction of *CYP1A1* mRNA expression caused by atRA was blocked by 1 μ M AGN193109. HepG2 cells were pretreated with 1 μ M AGN193109 (open squares) or DMSO (filled diamonds) for 1 h, and then exposed to 1–100 nM atRA for 20 h. After incubation, the

CYP1A1 mRNA levels were measured by real time PCR using *GUSB* mRNA as the endogenous control. Each value represents the mean \pm SD of three wells. * indicates significance at the $p < 0.05$ level (Student's *t*-test) between the Ct values of DMSO and AGN193109-pretreated cells.

B; RARA siRNAs suppressed RAR α mRNA expression and the inhibitory effect of atRA on CYP1A1 mRNA expression. HepG2 cells were transfected with 50 nM control siRNA (siNC, open columns) or siRNA for RAR α (siRARA, filled columns). Thirty-six hours after transfection, cells were exposed to 100 nM atRA or 0.1% DMSO for 20 h. After incubation, the *CYP1A1* mRNA levels were measured by real time PCR using *GUSB* mRNA as the endogenous control. Each value represents the mean \pm SD of three wells. * indicates significance at the $p < 0.05$ level (Student's *t*-test) between the Ct values of siNC and siRARA-transfected cells.

Figure 3. The inhibitory effect of atRA on CYP1A1 transactivation was dependent on XRE rather than RARE1A1 sequences.

HepG2 cells were transiently transfected with 3 types of the pGL3-promoter vector containing natural (filled diamonds, A and B), RARE-mutated regions (open triangles, A), or four XRE-deleted (opened squares, B) enhancer and promoter regions of human CYP1A1

with the control vector, pRL-SV40. After 24 h incubation in normal growth media, cells were treated with 0.1–100 nM atRA or 0.1% DMSO for 24 h. Transactivation ability was measured using a Dual-Glo Luciferase assay kit (Promega). Each value represents the mean \pm SD of three wells. * indicates significance at the $p < 0.05$ level (Dunnett's *t*-test) between the Ct values of controls and atRA-treated cells.

Figure 4. The recruitment of ARNT was greatly inhibited by atRA.

HepG2 cells were exposed to 1–100 nM atRA or 0.1% DMSO for 20 h. A ChIP assay was performed with either anti-AHR or anti-ARNT antibody, or with control normal rabbit IgG as described in the Materials and Methods section. The precipitated chromatin was analyzed using primers specific for the sequences located at –988 to –754 bp from the human CYP1A1 transcription start site.

Figure 1

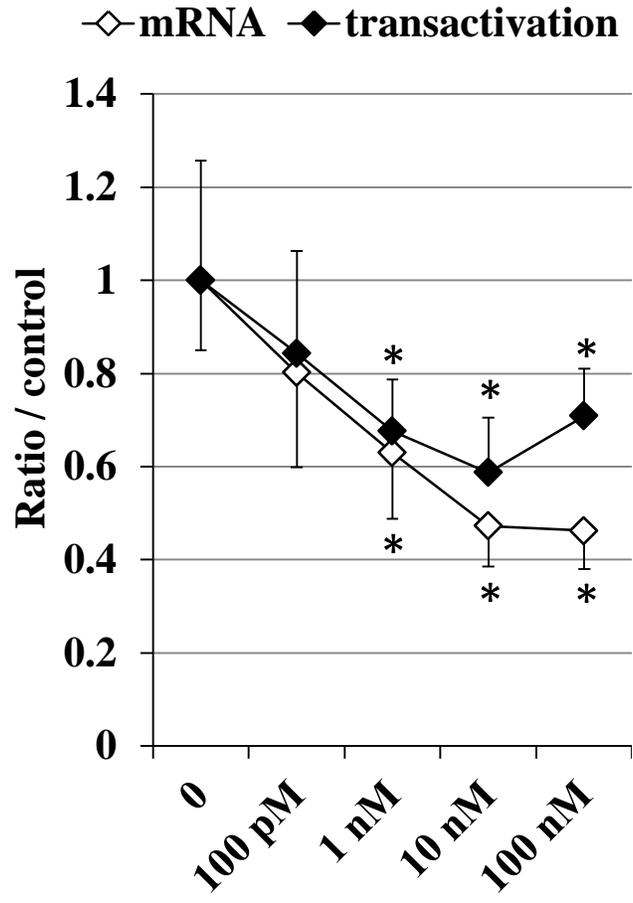
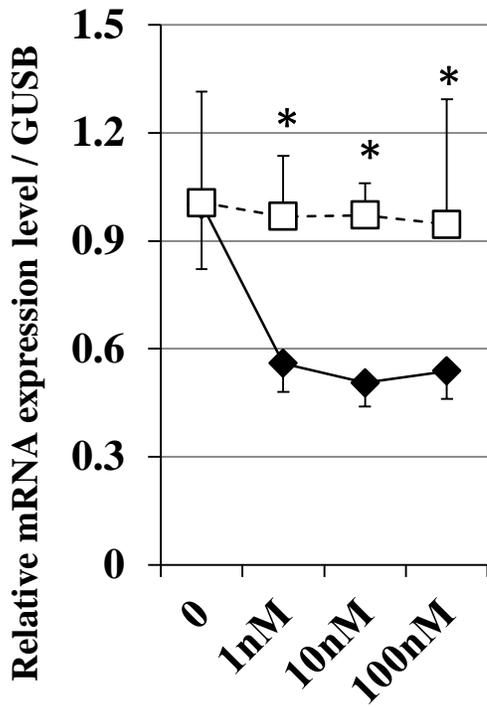


Figure 2

A. atRA vs. RAR antagonist

◆ DMSO - □ - 1 μ M AGN193109



B. atRA vs. siRNA for RAR α

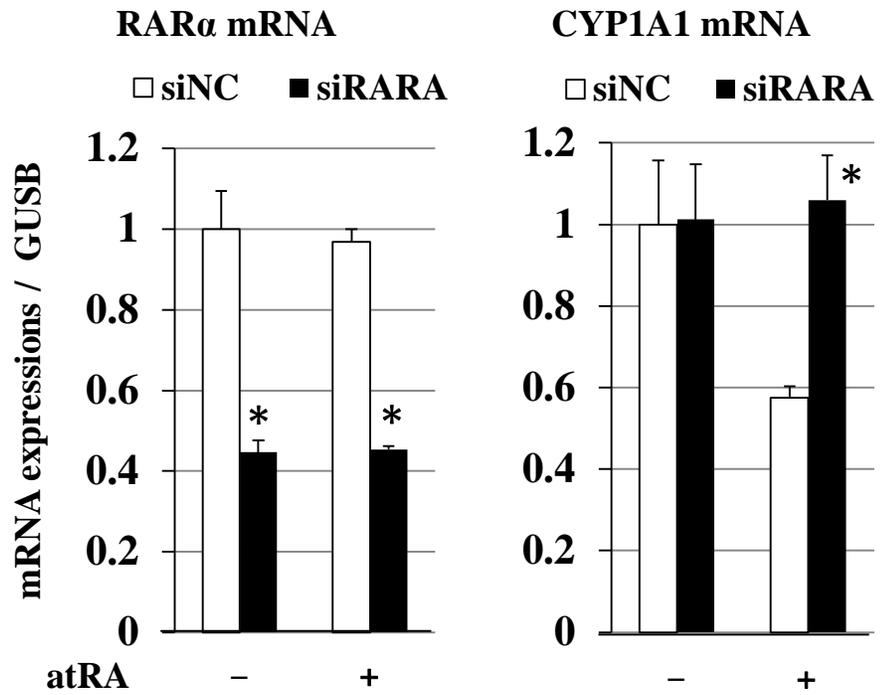


Figure 3

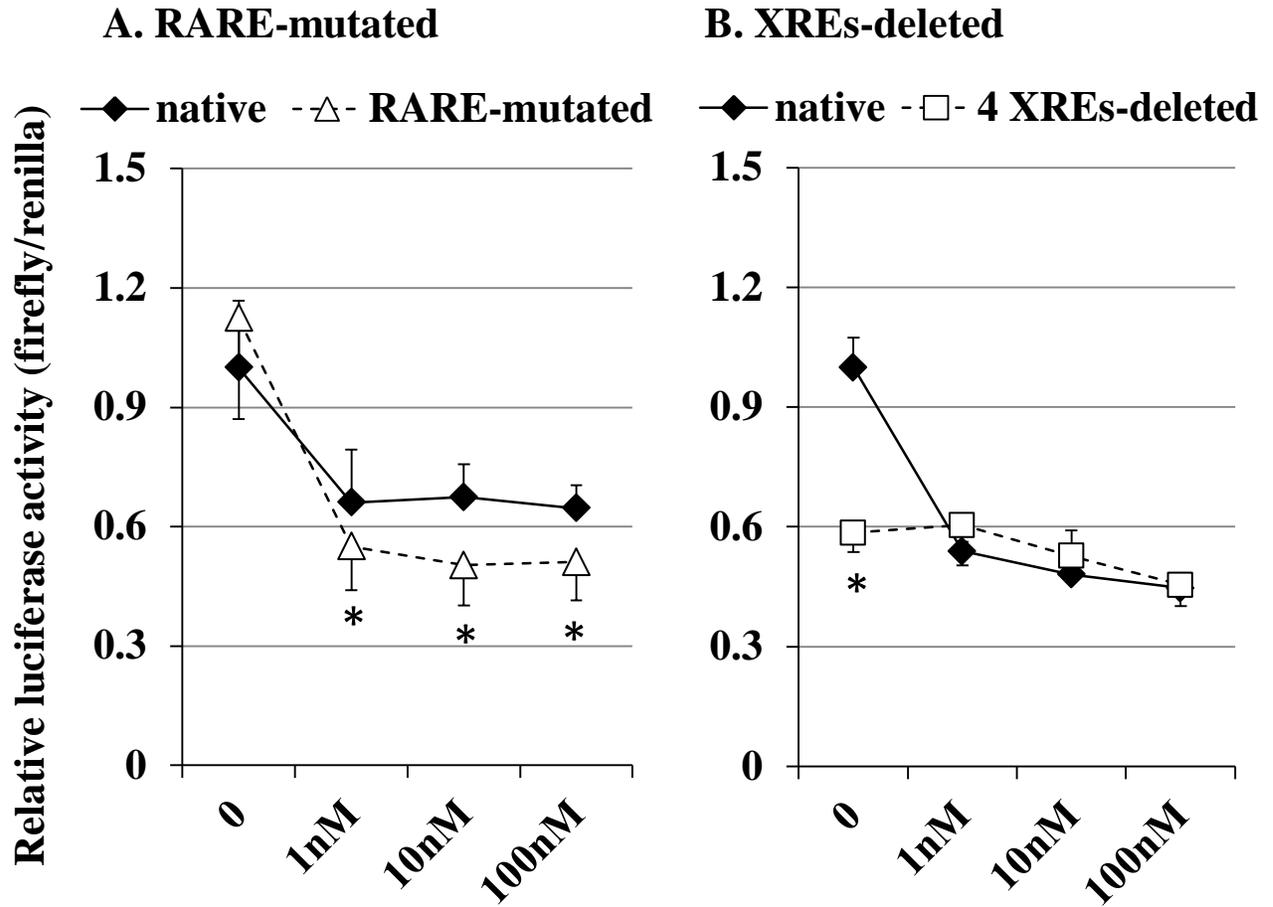


Figure 4

